Potential antibacterial activity of Botryosphaeria rhodina: Mangrove Xylocarpus granatum J. Koenig. derived fungal endophyte

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ABSTRACT
Exploring the secondary metabolites with excellent biological activity and pharmacy value from mangrove-derived fungi has attracted great attention. This study aims to isolate endophytic fungi that exhibit antimicrobial activity from Mangrove plants in Siput River Estuaries, Bengkalis Regency, Riau Province, Indonesia. Fifteen isolates of fungal endophytes have been isolated from the root of different Mangrove species, i.e., Aegiceras sp., Lumnitzera racemosa Wild., Avicennia marina (Forssk.) Vierh., Laguncularia racemosa (L) C. F. Gaertn., Sonneratia ovata Backer., Kandelia candel (L) Druce., and Xylocarpus granatum J. Koenig. In vitro antagonism test was performed for all isolated endophytes against selected pathogenic bacteria. Ten of fifteen endophytes were able to inhibit at least one pathogen with a diameter of inhibition zones ranging from 7,62 ± 0,55 to 18,20 ± 0,98 mm. Three fungi (F15, F2, and F5) with the highest antibacterial activity were subjected to molecular characterization based on the ribosomal region of the internal transcribed spacer by PCR amplification using ITS4 and ITS5 primers. The most potential isolate (F15 from Xylocarpus granatum J. Koenig) showed 99.82% similarity with Botryosphaeria rhodina isolate P130. Its ethyl acetate extract was found positive for terpenoid, phenolic, and flavonoid presence. F2 and F5, originated from L. racemosa Wild. and Avicennia marina (Forssk.) Vierh., had 100% similarity with Fusarium equiseti isolate FUS-34-2 and Aspergillus fumigatus strain DTO 402-H1, respectively. All endophytic fungi are recorded for the first time in Riau Mangrove.

INTRODUCTION
Antibiotic resistance is a worldwide phenomenon. Effective antibiotics are essential for preventing and treating infections [1–3]. Moreover, as a leading cause of morbidity, foodborne infections are among the most severe and expensive public health concerns worldwide. Thus, new antimicrobial compounds are needed to extend food shelf life, reduce food deterioration, and increase food safety [4,5].

Recently, natural products have been developed as an option for synthetic drugs due to their adverse side effects. Numerous plant metabolites have been widely used to cure several diseases [6,7]. Nonetheless, several key medicinal plants are gradually extinct due to overharvesting to meet market demands on new bioactive compounds. Therefore, scientists have begun working with endophytes in recent years as they can produce biomedically essential phytocompounds [8–11].

Mangroves, primarily tropical vegetations that thrive in harsh environmental conditions, support a variety of endophytes [12,13]. They are well known as sources of antibacterial [14,15], anticancer [16], antioxidant [8,17], antifungal [18,19], antiviral [12], enzyme activation and inhibition [20,21], immunosuppressive [22,23], anti-inflammatory [24,25], and antifeedant [12]. Indonesia is home to 20% of mangroves globally.
(3.2 million hectares), with 45 mangrove species. Furthermore, the province of Riau alone is responsible for approximately 143 billion hectares of this forest [26–28]. Although this archipelago contains the world’s most valuable mangrove ecosystem, only a few studies have been published on exploring Indonesia’s mangrove-derived endophytes for their bioactive compounds of medicinal significance. Nevertheless, no study has been undertaken to explore the potential endophytes derived from mangroves in Riau Province.

Antimicrobial activity from endophytic fungi isolated from mangroves in Indonesia was rarely studied. We have published promising bacterial and fungal endophytes as sources of antimicrobial compounds derived from mangroves in the coastal area of Riau Province [14,29]. In this study, we explored for the first time the diversity of fungal endophytes from mangroves in Siput River Estuaries that exhibit antibacterial activity and their molecular identification through ITS rDNA amplification.

**MATERIAL AND METHODS**

**Sampling site and isolation of culturable endophytes**

Eight healthy mangroves with different characters were chosen randomly from the estuaries of Siput River, Bengkalis Regency, Riau Province, Indonesia (1°15ʹ59” LU; 102°8ʹ31” BT). Plant specimens (roots, leaves, twigs, and flowers/fruit) were taken, wrapped in aluminum foil, and sent to University Herbarium for species identification. The root samples for endophytic fungi isolation were brought to laboratory in separate sterile polyethylene bags in a surface-sterilized box with dried ice. Endophytic fungi were then isolated from each sample’s root immediately after reaching the lab using a standard protocol as described in our previous work [29].

**In vitro antagonism test**

All isolated endophytes were screened for in vitro antagonism activity against selected pathogenic bacteria to reveal their potential as an antimicrobial compound(s) source. Bacterial indicators used in this analysis including Gram-positive (Staphylococcus aureus ATCC29213 and Bacillus subtilis ATCC11774) and Gram-negative (Escherichia coli ATCC35218, Vibrio parahaemolyticus, and Vibrio alginolyticus), the culture collection of the Biochemistry and Molecular Biology Laboratory, University of Riau. A plug of 5-day-old culture (6 mm diameter) of each fungal endophyte was inoculated on the surface of Mueller–Hinton Agar (MHA; Oxoid, Hampshire, England) containing each pathogenic bacteria (10⁶ CFU/plate) and incubated overnight at 37°C. MHA plates containing each pathogen without fungal endophyte were used as the negative control. All procedures were carried out in triplicate. The diameter of the inhibition zone (in mm) was measured using a caliper after 24 hours of incubation. Endophytes showing broad-spectrum antibacterial activity with high inhibition zone were then identified based on morphology on potato dextrose agar (PDA; Oxoid, Hampshire, England) plate and their ribosomal RNA gene’s internal transcribed spacer (ITS) regions using universal primers (IDT, Coralville, IA).

**ITS region amplification and sequencing**

Each isolated endophyte was cultured on potato dextrose agar. Genomic DNA from each endophyte was isolated using Geneaid GBYB100 Presto Mini gDNA Yeast Kit. Amplification of ITS region was performed using 10 µM universal primers ITS4 (ITS4-R 5’- TCC TCC GCT TAT TGA TAT GC-3’) and ITS5 (ITS5-F 5’- GAA AGT AAA AGT CGT AAC AAG G-3’) at 50.5°C annealing temperature for 45 seconds. Negative control (absence of DNA template) was used to detect DNA contamination.

Sequencing was carried out at Genetica Science Indonesia. The obtained sequences were compared to those available in GenBank via the Basic Local Alignment Search Tool (BLAST) for final identification. Multialignment and construction of sequence differences for edited sequences obtained in this study and sequences from the GeneBank database at the National Center for Biotechnology Information Nucleotide Sequence database were performed using BioEdit software. The phylogenetic tree was built using the neighbor-joining algorithm and bootstrapping of 1,000 replications by MEGA6.

**Phytochemical analysis**

For this analysis, small-scale fermentation of Botryosphaeria rhodina was first carried out, followed by extraction using ethyl acetate. Fermentation in solid rice medium and secondary metabolites extraction was performed according to the procedure described by Kjer et al. [30]. Solid rice medium was prepared by adding 200 g rice with 210 ml water in a 2,000 ml Erlenmeyer flask and sterilized at 121°C for 15 minutes. A plug with a size of ~1.5 × 1.5 cm was taken from a pure fungal strain that was freshly grown and covered the surface of the inoculated PDA plate and transferred into an Erlenmeyer flask containing sterile solid rice medium. The culture was incubated at room temperature for 10 days.

The culture medium containing the mycelium was then cut into small pieces, extracted with ethyl acetate, and filtered under vacuum using a Buchner funnel. The ethyl acetate extract was then subjected to phytochemical analysis to determine the presence of alkaloids, terpenoids, steroids, saponins, phenols, and flavonoids in accordance with Harborne [31] and Akhtar et al. [32].

**Statistical analysis**

Antibacterial activity was expressed as inhibition zones formed around the endophytes and presented as mean ± standard deviation of three replicates. One-way analysis of variance was run using Minitab® software version 19 (Pennsylvania, USA) to determine the significant differences, followed by Tukey’s pairwise comparison at α = 5%.

**RESULTS**

**Isolation of culturable endophytes from the roots of mangroves**

Figure 1 shows the sampling location of Siput River estuaries in Siak Kecil Regency, Bengkalis. As shown in Table 1, a total of 15 fungal endophytes were isolated from
the roots of eight mangrove samples, which were identified as *Aegiceras* sp. (1 isolate), *Lumnitzeria racemosa* Wild. (2 isolates), *Avicennia marina* (Forssk.) Vierh. (6 isolates), *Laguncularia racemosa* (L) C. F. Gaertn. (1 isolate), *Sonneratia ovata* Backer. (3 isolates), *Kandelia candel* (L) Druce. (1 isolate), and dan *Xylocarpus granatum* J. Koenig (1 isolate).

**Antagonistic activity of endophytes against Gram-positive and Gram-negative bacteria**

Table 1 depicts the result of *in vitro* antagonism activity of 15 isolated endophytes. The mean of inhibition zones was compared among endophytes against each pathogen. Out of 15 isolates tested, ten isolates actively inhibited the growth of at least one pathogen, and *E. coli* was found to be the most inhibited. The highest inhibition against *V. alginolyticus* was shown by F15, while F2 was found to be the most potential isolate to produce a compound to inhibit the growth of *B. subtilis*. Isolates F15, F2, and F5 were the most potential producers for compounds with antimicrobial activity as they exhibited high inhibition against both Gram-positive and Gram-negative bacteria.

**ITS region amplification and sequencing**

Our results showed that PCR analysis using ITS1 and ITS4 primers generated a single band of ~550–600 bp. The original neighbor-joining tree with 1,000 bootstrap replications is shown in Figure 3. Based on sequence similarity as determined by the BLAST program in the GenBank database, and according to the phylogeny tree, the closest species of isolates F2, F5, and F15 is *Fusarium equiseti* isolate FUS-34-2 (Accession no. MH879250.1; 100%), *Aspergillus fumigatus* strain DTO 402-H1 (Accession no. MT316338.1; 100%), and *B. rhodina* isolate P130 (Accession no. EF423547.1; 99.82%), respectively.

**Phytochemical analysis**

Table 2 shows the phytochemical analysis data of the secondary metabolites in the *B. rhodina* extract. Our finding confirmed the presence of terpenoid, phenolic, and flavonoid.

**DISCUSSION**

Endophytes are microorganisms that live inside plant tissues without harming the host plant. These microorganisms can be bacteria, fungi, or viruses and can exist in various plant tissues, including leaves, stems, and roots. Endophytes have been demonstrated to contribute to plant growth, health, and resilience to various biotic and abiotic stresses. Endophytes and their correlation with antimicrobial activity in numerous plant species, including mangroves, have been extensively researched.[33, 34]. Mangroves are a group of salt-tolerant trees that grow in the intertidal zones of tropical and subtropical coasts. Mangroves have diverse environmental impacts, including protecting soils from floods and cyclones, preserving the integrity of river banks, and promoting biodiversity. These ecosystems also yield various natural products with potential benefits and commercial value. Furthermore, mangroves have a history of providing medicinal and food products. The natural products sourced from mangroves have various applications in areas such as agriculture, aquaculture, reforestation, and infrastructure.[35].

Mangroves have been proven to harbor various endophytes, many of which produce antimicrobial compounds.
The unique environmental conditions found in mangroves, such as high salinity, temperature fluctuations, and limited nutrient availability, may have led to the evolution of unique endophyte communities with specialized metabolic capabilities, including the production of antimicrobial compounds [36,37].

Riau Province is located in the central part of Sumatra Island, Indonesia. This province covers 8,702,366 hectares of total area, with Pekanbaru as the capital city. As reported by the Forestry Ministry service of Riau, this province has 138,434 hectares of mangrove forest, constituting 21.6% of Indonesia’s total mangrove area. Bengkalis is one of 10 regencies in Riau Province with 21,981 hectares of mangrove forest. However, no scientific paper reports the exploration of endophytes from Riau Mangrove as a potential source of antimicrobial compounds. For the first time, this work discusses the endophytes of mangroves from Siput River estuaries in Siak Kecil Regency, Bengkalis, Indonesia. This area is located around the border of the Malacca Strait (Fig. 1).

The results of the *in vitro* antagonism test showed that ten isolates were able to inhibit the growth of at least one pathogen. Isolate F15 was the best isolate based on the One-Way Analysis of Variance (*α* = 5%) in comparing the mean of clear zone inhibition diameter toward all pathogens tested, followed by F2 and F5. Figure 2 shows the macroscopic of those isolates in potato dextrose agar and their microscopic character.

<table>
<thead>
<tr>
<th>Table 1. Mangrove samples and isolated fungal endophytes’ antimicrobial activities.</th>
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<tr>
<td><strong>Mangrove sample (species)</strong></td>
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<tr>
<td>Mangrove 1 (Aegiceras sp.)</td>
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<td>Mangrove 2 (Lumnitzera racemosa Wild.)</td>
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<td>Mangrove 3 [Avicennia marina (Forssk.) Vierh.]</td>
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<td>Mangrove 4 [Laguncularia racemosa (L.) C. F. Gaertn.]</td>
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<td>Mangrove 5 [Sonneratia ovata Backer.]</td>
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<td>Mangrove 6 [Avicennia marina (Forssk.) Vierh.]</td>
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<td>Mangrove 7 [Kandelia candel (L.) Druce.]</td>
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<td>Mangrove 8 (Xylocarpus granatum J. Koenig.)</td>
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<td>Mangrove 9 [Avicennia marina (Forssk.) Vierh.]</td>
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<td>Mangrove 10 [Sonneratia ovata Backer.]</td>
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<td>Mangrove 11 [Kandelia candel (L.) Druce.]</td>
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<td>Mangrove 12 [Avicennia marina (Forssk.) Vierh.]</td>
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<td>Mangrove 13 [Sonneratia ovata Backer.]</td>
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<td>Mangrove 14 [Kandelia candel (L.) Druce.]</td>
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<tr>
<td>Mangrove 15 [Avicennia marina (Forssk.) Vierh.]</td>
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</table>

*Mean ± standard deviation.
**Means that do not share a letter in the same column are significantly different (*α* = 5%).
**Bold:** Endophytic fungi exhibited the highest diameter of inhibition against pathogens.

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Linda et al. / Journal of Applied Pharmaceutical Science 14 (02); 2024: 136-143

Grew vigorously on the surface of PDA. In 3–4 days, the surface of the PDA has been fully covered by its greyish-white aerial mycelia. These data indicated the identity of genus *Fusarium*, *Aspergillus*, and *Botryospharia*.

Endophytes enhance the physiology of their host plants, which results in improved health compared to plants without them [38]. This is because endophytes release substances such as cytokines, phytohormones, and other plant growth-promoting compounds, which boost the growth of host plants’ growth directly or indirectly. Moreover, endophytes can produce compounds that were formerly considered synthesized by plants. This is probably due to the horizontal gene transfer from plant to endophyte genome or vice versa [39]. Numerous endophytes can produce a variety of bioactive metabolites that can be used as therapeutic agents against various diseases, either directly or indirectly. Furthermore, diverse microorganisms in plant tissues enable microbial interactions such as quorum sensing, resulting in the production of metabolites [40].

Mangrove-derived endophytes are rich sources of various pharmacologically active metabolites, as mangroves produce chemically unique secondary metabolites that are diverse in nature [16,35]. The presence of alkaloids has been reported from the mangrove endophytic fungus *Phomopsis* sp. SYSUQYP-23 and *Didymella* sp. CYSK-4 [25,41]. Other researchers found that endophytes from mangroves produced coumarins, terpenoids [42], and polyketides [43,44].

Advances in molecular genetics allowed the incorporation of DNA analysis into the taxonomy. In this study, a molecular approach for species differentiation was performed to amplify the ITS region, a powerful tool to characterize fungal diversity that is located between the highly conserved genes coding for 18S and 28S rRNA [45,46]. The regions are characterized by a high degree of fungi heterogeneity and have been reported to be useful in mold phylogenetic and taxonomic analyses [47,48]. Our finding showed amplicons with a size of ~550–600 bp. The 16S rRNA sequence-based phylogenetic tree analysis (Fig. 3) revealed that the lineages of the isolates could be divided into three main groups, representing different genera, i.e., *Botryosphaeria*, *Fusarium*, and *Aspergillus*. Nucleotide BLAST results showed that the F2, F5, and F15 are *Fusarium equiseti*, *A. fumigatus*, and *B. rhodina*, respectively. All species found in this study were previously reported as endophytic fungi. The genus *Fusarium* is widely distributed throughout the world in the form of pathogenic and nonpathogenic strains. *Fusarium equiseti* is an endophyte fungus that has been found in rice [49], medicinal plant *Cananga odorata*, barley roots [50], and *Limonium cossonianum* from a coastal salt marsh [51]. The presence of the genus *Fusarium* in mangroves has been reported from *Kandelia candel* and *Podophyllum hexandrum* [35]. The genus *Fusarium* is a potential fungi producer of novel antibiotics [50]. Our finding showed that isolate F2, *Fusarium equiseti* from mangrove *L. racemosa* Wild, had a bigger clear zone toward *B. subtilis* ATCC11774 than *E. coli* ATCC35218. This result is in agreement with other researchers who reported that *Fusarium equiseti* has better inhibition against gram-positive bacteria due to the presence of an antibiotic like “enniatins.”
Aspergillus species have been defined as endophytes, saprophytes, parasites, and human pathogens, all of which exhibited a high propensity for producing secondary metabolites with diverse chemical structures and bioactivities [52]. In a previous report, endophytic A. fumigatus was isolated from mangrove Someratia griffithii Kurz in West Sumatra, Indonesia [15], and in inland salt marshes [51]. In the present study, A. fumigatus was isolated from the mangrove Avicennia marina (Forssk.) Vierh. and denoted the potential of broad-spectrum activity as it suppressed the growth of E. coli ATCC35218, V. parahaemolyticus, and B. subtilis ATCC11774. Our finding is in line with previously published papers that reported the wide-range activity of metabolites from A. fumigatus endophyte, for instance, heterocyclic alkaloids as antimicrobial agents [53,52], immunosuppressive agents [22]. Much attention has been paid to a particular group of endophytic fungi belonging to Botryosphaeria in recent years. Our study found that the most potential isolate, isolate F15, is B. rhodina from mangrove Xylocarpus granatum J. Koenig. Botryosphaeria rhodina has been reported previously in Mangrove Kandelia candel [54] and the leaves of Garcinia mangostana [55]. Other researchers isolated the group of Botryosphaeria sp. in the medicinal plant Bidens pilosa [56].

Metabolites from Botryosphaeria species are some notable classes of chemical moieties, including naphthalenones, lactones, polyketides, diterpenoids, benzofuran derivatives, and exopolysaccharides [55] that have potential antibacterial properties [57]. Antibacterial activity of B. rhodina is probably due to the presence of antibacterial primin, as well as several novel compounds such as alkaloids, coumarins, ceramides, lactones, diterpenoids, benzofuran derivatives, meroterpenoids, polyketides, and polysaccharides [54].

The results of the antagonistic test against multiple pathogenic bacteria showed that B. rhodina possesses a high degree of inhibiting ability. Therefore, a small-scale fermentation followed by ethyl acetate extraction was performed. As shown in Table 2, this extract was subjected to identification testing for secondary metabolite compounds. These tests revealed that B. rhodina can synthesize secondary metabolite compounds belonging to the terpenoid, phenolic, and flavonoid classes. Botryosocoumarin A, a phenolic compound isolated from B. rhodina, demonstrated antibacterial activity, as reported by Ju et al. [58]. Botryorhodines A–D, which are also phenolic compounds, have antimicrobial activity, according to Abdou et al. [56]. Based on the results of this phytochemical analysis, a preliminary conclusion can be drawn that the antibacterial activity of B. rhodina is derived from secondary metabolites such as phenolic compounds, and this preliminary result can serve as a guide for future research on isolating secondary metabolites from this species.

### Table 2. Phytochemical analysis data of B. rhodina extract.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Chemical reagent</th>
<th>Result</th>
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<tbody>
<tr>
<td>Alkaloid</td>
<td>Meyer and Dragendorf</td>
<td>-</td>
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<tr>
<td>Steroid</td>
<td>Liebermann–Burchard</td>
<td>-</td>
</tr>
<tr>
<td>Terpenoid</td>
<td>Liebermann–Burchard</td>
<td>+</td>
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<tr>
<td>Saponin</td>
<td>H,O</td>
<td>-</td>
</tr>
<tr>
<td>Fenolik</td>
<td>FeCl₂</td>
<td>+</td>
</tr>
<tr>
<td>Flavonoid</td>
<td>Mg-HCl</td>
<td>+</td>
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</tbody>
</table>

### CONCLUSION

Endophytes have been widely recognized as important components of the mangrove ecosystem and have been shown to play a crucial role in plant growth, health, and disease resistance. Isolation of endophytes from mangroves has revealed a diverse community of microorganisms that produce a variety of antimicrobial compounds with activity against a range of pathogens. This study has identified three endophytic fungi from Mangrove L. racemosa Wild., Avicennia marina (Forssk.) Vierh., and Xylocarpus granatum J. Koenig, which are potential sources of wide spectrum antibacterial agents. These fungi have close relatedness with Fusarium equiseti isolate FUS-34-2 (100%), A. fumigatus strain DTO 402-H1 (100%), and B. rhodina isolate P130 (99.82%), respectively. The ethyl acetate extract of B. rhodina, the most potential isolate, was found positive for the presence of terpenoid, phenolic, and flavonoid. This report highlights the importance of ongoing efforts to isolate, study, and understand the diversity and functions of endophytes in mangroves and other plant species.

### Declaration of Competing Interest

The authors declare that no competing personal and/or financial interests influenced the works reported in this paper.

### ACKNOWLEDGMENT

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### AUTHOR CONTRIBUTIONS

All authors made substantial contributions to conception and design, acquisition of data, or analysis and interpretation of data; took part in drafting the article or revising it critically for important intellectual content; agreed to submit to the current journal; gave final approval of the version to be published; and agree to be accountable for all aspects of the work. All the authors are eligible to be an author as per the international committee of medical journal editors (ICMJE) requirements/guidelines.

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### CONFLICTS OF INTEREST

The authors report no financial or any other conflicts of interest in this work.
ETHICAL APPROVALS
This study does not involve experiments on animals or human subjects.

DATA AVAILABILITY
All data generated and analyzed are included in this research article.

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